Two Gq Class G Proteins Are Expressed in Human Keratinocytes

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G proteins link many cell surface receptor generated signals to activation of multiple cellular processes in all tissues. There is specificity in the receptor interaction with the G protein and in the interaction of the specific G protein with different effector molecules. The purpose of this study was to determine some of the biologically relevant G proteins in keratinocytes. The Gα subunit of the heterotrimeric G protein was investigated because much of the biologic activity and the receptor specificity resides there. A polymerase chain reaction strategy was used that amplified multiple Gα gene segments between conserved primer sites from keratinocyte first strand cDNA. Two Gq class G proteins, Gα16 and Gαy, were identified. Using northern analysis and *in situ* hybridization, mRNA of both of these genes were detected in keratinocytes in culture and in epidermal keratinocytes. Gαy was expressed in multiple other cell types and tissues, but Gα16 was restricted in expression to keratinocytes and keratinocyte-derived adnexal structures in the skin. Gα16 has previously been reported to be limited in expression to hematopoietic cells. The physiologic receptor to which it couples in neutrophils is reported to be a C5a receptor. The receptor to which it couples in keratinocytes has not been elucidated but by analogy may be another chemokine receptor. We hypothesize that Gα16 is an important conduit for responses to inflammatory signals in keratinocytes. *Key words: chemokine/Gα16/Gαy/signal transduction. J Invest Dermatol 109:645–649, 1997*

G proteins are ubiquitous, heterotrimeric [α (36–52 kDa), β (35–36 kDa), and γ (8–10 kDa)] signal transducers between seven transmembrane domain receptors and cytoplasmic effector molecules. They are part of a superfamily of guanosine triphosphate (GTP)-binding proteins that exhibit a conserved structure and share a common molecular switch mechanism mediated by the binding and hydrolysis of GTP (Bourne *et al*, 1990, 1991). G proteins regulate critical biologic processes in the skin and other tissues via transduction of signals from various neurotransmitters, hormones, and inflammatory mediators to specific effector molecules in the cell. Relevant G proteins in keratinocytes have not been directly elucidated.

Each G protein subunit contributes to the specificity and the regulation of signal transduction; however, we investigated the Gα subunit because it contains the receptor binding site, the GTP binding site, the GTPase activity, and a high affinity binding site for Mg^{2+} that is required for the formation of the activated GTP bound molecule. At least 16 genes encode mammalian Gα subunits that have been grouped by amino acid sequence homologies into families: Gs, Gi, Gq, and G12 (Simon *et al*, 1991). The Gq class of Gα subunits is potentially important for some types of inflammatory skin disease because they are known to transduce signals from many inflammatory mediators. Gq class G proteins identified include Gαq, Gαy/Gα11, Gα14/G_L1α, and Gα15/Gα16. Gq class G proteins have been shown to activate phosphoinositide-phospholipase C-β isoenzymes (PLC-β) that stimulate the hydrolysis of the membrane lipid phosphatidylinositol 4,5-biphosphate to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG) intracellular second messengers (Wu *et al*, 1992). IP3 increases intracellular calcium and induces calcium-dependent protein kinases. DG activates protein kinase C. Subsequent phosphorylation of proteins through intracellular signalling pathways including the mitogen-activated protein kinase cascade leads to nuclear gene activation and cellular responses (Russell and Hoeffler, 1996).

In this study we use a polymerase chain reaction (PCR) cloning strategy to identify some of the Gα genes expressed in keratinocytes. We have identified two members of the Gq family and one member of the Gi family of Gα genes in keratinocytes. One of the Gq genes, Gα16, is a rarely expressed gene that had been thought to be limited in expression to cells of the hematopoietic lineage. We demonstrate by northern analysis and by *in situ* hybridization that Gα16 is expressed in keratinocytes, but not in melanocytes, fibroblasts, or microvascular cells derived from normal skin. Because Gα16 is known to couple chemokine receptor signals to PLC-β pathways, we hypothesize that Gα16 may participate in inflammatory reactions in the skin.

MATERIALS AND METHODS

PCR generation of G protein probes The various Gα subunits contain five regions of conserved amino acid sequences designated A, C, E, G, and T (Pupillo *et al*, 1989). Degenerate oligonucleotide 17mer primers to region A and C were synthesized with a *NotI* restriction site and 3 nt edge added at the 5′ ends to facilitate cloning. All possible nucleotide sequences to the region "A" peptide sequence (Gly, Glu, Ser, Gly, Lys, and Ser) were synthesized and pooled and resulted in a degeneracy of 756-fold. All possible nucleotide sequences to the region "C" peptide sequence (Arg, Gln, Gly, Gly, Val, and Asp) were synthesized and pooled and resulted in a degeneracy of 512-fold. These primers have previously been used to amplify Gα subunits from murine

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Abbreviations: DG, diacylglycerol; EDTA, ethylenediamine tetraacetic acid; GTP, guanosine triphosphate; IP3, inositol 1,4,5-triphosphate; PCR, polymerase chain reaction; PLC-β, phosphoinositide-phospholipase C-β; SSC, sodium citrate/chloride buffer.

lymphocytes demonstrating their cross-species utility (Rock *et al*, 1995). Using sequences from conserved regions for primers allows amplification of multiple expressed G proteins. Because the area of greatest divergence between conserved regions in the Gα amino acid sequence is the ≈160 amino acids between A and C, their choice as primers allows the potential to generate unique cDNA clones that will be easily identified by DNA sequencing, and will be appropriate as specific probes for blotting. The template for the PCR reaction was prepared as follows. Total RNA was harvested by guanidinium isothiocyanate single step extraction method from cultured neonatal human keratinocytes (Chomczynski and Sacchi, 1987). The mRNA was separated by oligo dT cellulose column chromatography and first strand cDNA prepared with reverse transcriptase using random hexonucleotide primers (cDNA Synthesiz Plus, Amersham, Arlington Heights, IL). The PCR reaction was performed in a DNA thermocycler (Perkin-Elmer Cetus, Norwalk, CT) with Taq polymerase (GeneAmp DNA amplification kit) using step cycling (denature –94°C, 1 min; anneal –60°C, 2 min; extension –72°C, 2 min; 30 cycles). The PCR product was purified by 1% low melt agarose gel electrophoresis and column chromatography (NACS, Bethesda Research Laboratories, Grand Island, NY). Low molecular weight contaminants were removed by ultrafiltration (Ultrafree-MC, Nihon Millipore Kogyo K.K., Yonezawa, Japan). The purified product was ligated into pBluescript at the *Not* 1 cloning site and the ligation mixture used to transform *Escherichia coli* (DH1). Eleven plasmids with inserts identified by plasmid DNA mini-preps were evaluated by dot blot cross-hybridization that revealed three unique Gα clones (data not shown) for analysis.

Sequencing reaction Unique cDNA clones were sequenced by the didioxy chain termination method with ^{35}S dATP (Sequenase, United States Biochemical, Cleveland, OH). Sequencing reactions were separated on an 8% polyacrylamide gel (Accugel 40, National Diagnostics, Manville, NJ), dried, and exposed to x-ray film for 1 d. Reactions were read directly from a gel reader (IBI, New Haven, CT) connected to a sequencing program (MacVector). Sequences were aligned to DNA databases via Entrez (National Center for Biotechnology Information, Bethesda, MD).

Human keratinocyte, melanocyte, microvascular endothelial cell, and fibroblast isolation and culture A modification of the technique of Boyce and Ham was used for keratinocyte isolation (Boyce and Ham, 1985). After neonatal circumcision, discarded foreskins were placed into Hanks' balanced salt solution with calcium chloride 0.185 g per liter, magnesium chloride 0.0976 g per liter, and 5 × Pen/Strep/AmphoB (Sigma, St Louis, MO). Foreskins were stored at 4°C until cell harvesting. At the time of harvesting, foreskins were washed in 70% ETOH for 2–3 min and rinsed in solution A (7.149 g HEPES, 1.802 g glucose, 0.224 g KCL, 7.697 g NaCl, 0.268 g $Na_2HPO_4.7H_2O$, 1 ml of 1000 × phenol red stock dissolved in 1 liter total volume high purity water with pH adjusted to 7.4 and filtered through 0.2 mm pore size). The foreskins were cut into 3 mm strips and transferred to a dispase solution (Dispase II, Boehringer, Indianapolis, MN) 1.2 units per ml in sol A and incubated overnight at 4°C. The epidermis was pealed and incubated in trypsin/ethylenediamine tetraacetic acid (EDTA) solution (trypsin 0.025%, EDTA 0.01%, pH 7.4) with gentle shaking for 4 min. The reaction was stopped with heat inactivated 10% newborn calf serum in sol A at 1:1 with epidermal suspension. Cells were gently centrifuged and resuspended in keratinocyte growth medium with bovine pituitary extract (Clonetics, San Diego, CA) with 0.30 mM calcium added and plated directly into flasks at ≈6 foreskin/T25 (Falcon, Becton Dickinson). After the first day keratinocyte growth medium without additional calcium was used. Cell medium was changed every 3–4 d and cells split at 50% confluence. Melanocytes were harvested from primary, nonconfluent keratinocyte cultures. Melanocytes were released with direct microscopic visualization by brief trypsinization of flasks (< 1 min, room temperature) that selectively releases the melanocytes prior to the keratinocytes. The melanocytes were then plated in melanocyte growth medium (Cascade, Portland, OH) and grown to confluence. By passage 2, melanocyte cultures were free of contaminating keratinocytes. Microvascular endothelial cells were harvested from neonatal foreskin as previously described (Kubota *et al*, 1988). Fibroblasts were obtained by incubating the dermal portion of the neonatal foreskin in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) with collagenase (Sigma C 9722, type 1 A S) at a final concentration of 1000 units per ml, rocking at 37°C for 1.5 h. The suspension was spun, pelleted, resuspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and Pen/Strep/AmphoB (Sigma), and plated. Vigorous cultures were evident at 1 wk.

Northern blot analysis Ten to twenty micrograms of total RNA samples from keratinocytes, melanocytes, dermal microvascular endothelial cells, and fibroblasts were fractionated over formaldehyde-agarose gels, transferred to Nytran (Schleicher & Schull, Keene, NH) nylon membranes, and cross-linked (UV Stratalinker, Stratagene, La Jolla, CA). Membranes were prehybridized at 42°C for 1 h or greater in a solution of 50% formamide, 1 M NaCl, 10% dextran sulphate, 1% sodium dodecyl sulfate, and 200 mg yeast tRNA per ml

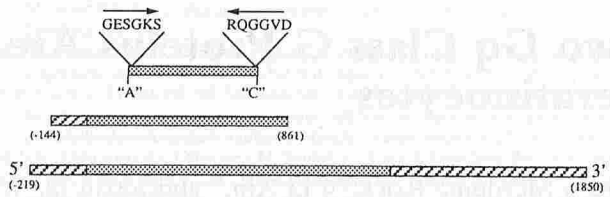

Figure 1. Relationship of PCR-cloned segment (*upper bar*) to the cDNA segment pulled from a keratinocyte library (*middle bar*) and to the full-length Gαl6 cDNA (*lower bar*). *Open bars*, open reading frame. Gαl6 GenBank accession no. = M63904.

and hybridized overnight at 42°C with 10–40 ng of randomly primed ^{32}P-labeled cDNA fragments, either Gα16 or Gαy PCR generated clones (specific activities = 10^8–10^9 cpm per mg). Probed membranes were washed under increasingly stringent conditions and exposed to XAR-2 film with one intensifying screen at –70°C for varying times. The quality of the RNA was determined by visualization of the ethidium bromide stained gels before and after transfer and reprobing of blots with β actin or β tubulin.

***In situ* hybridization** *In situ* hybridization was performed with ^{35}S-labeled riboprobe generated from Gα16 and Gαy PCR-generated cDNA clones with sense controls as previously described (Wilcox, 1993). Briefly, cryosections of normal archival baboon skin were pretreated with 4% paraformaldehyde, 1 mg proteinase K per ml (Sigma), and prehybridized in 100 ml hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 0.02% polyvinylpyrrolidine, 0.2% Ficoll, 0.02% bovine serum albumin, 10% dextran sulphate, and 10 mM dithiothreitol) at 42°C. The hybridization was started by adding 600,000 cpms of ^{35}S-riboprobe in a small volume of hybridization buffer into the prehybridization solution. The sections were then incubated at 55°C overnight. After hybridization, the sections were washed with 2 × sodium citrate/chloride buffer (SSC) (1 × SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0) with 10 mM β-mercaptoethanol and 1 mM EDTA, treated with 20 mg heat-treated RNase A per ml (Sigma), again washed in the same buffer, followed by a high stringency wash in 0.1 × SSC with 10 mM β-mercaptoethanol and 1 mM EDTA, at 55°C. The slides were then washed in 0.5 × SSC without β-mercaptoethanol and 1 mM EDTA and dehydrated in graded alcohols containing 0.3 M NH4Ac. The sections were dried, coated with NTB2 nuclear track emulsion (Kodak, Rochester, NY), and exposed in the dark at 4°C for 4–10 wk depending on the signal intensity. After development, the sections were counterstained with hematoxylin and eosin to aid in cell identification and viewed on a Leitz Aristoplan (Rockleigh, NY) photomicroscope equipped with polarized light epiluminescence to visualize the silver grains.

Library screening A human keratinocyte library (Amgai *et al*, 1991) was screened with ^{32}P-labeled PCR derived Gα16 cDNA. After tertiary screening and restriction mapping three overlapping clones were identified, the longest of which was sequenced.

RESULTS

Two Gq proteins were identified in keratinocytes by PCR cloning Eleven Gα gene segments of ≈450 nucleotides were amplified by PCR. By a series of southern dot blot cross-hybridization these were determined to represent three unique cDNA clones (data not shown). The three unique clones were sequenced and aligned to DNA databases. By sequence analysis they included two Gq genes and one Gi gene (which was not further analyzed). The Gq genes identified included Gα16 and Gαy. Gαy is widely distributed in human tissue and therefore its detection is not surprising; however, Gα16 is reported to be limited in expression to hematopoietic cell lineages (Amatruda *et al*, 1991). Therefore, to confirm the identity and expression of Gα16 in keratinocytes, the PCR-generated clone was used as a probe to screen a keratinocyte library. Three overlapping cDNA clones totaling 1005 nucleotides in length were identified and sequenced and found to be homologous to Gα16 containing a portion of the 5′ untranslated region and most of the open reading frame (**Fig 1**).

Gα16 was uniquely expressed in keratinocytes *in vitro* and has limited tissue distribution Northern analysis using the Gα16 and Gαy PCR-generated cDNA clones was performed on cells in culture derived from neonatal foreskin and including dermal fibroblasts, microvascular endothelial cells, melanocytes, and keratinocytes. Gα16 was expressed in keratinocytes with a message size of ≈2.2 kb, but not in

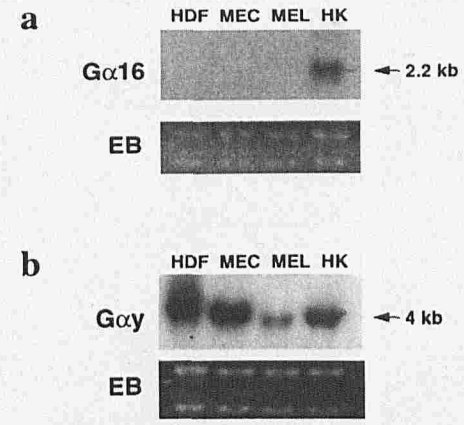

Figure 2. Northern analysis of cultured cells derived from neonatal foreskin. Total RNA was harvested from neonatal foreskin derived cells in culture, fractionated over formaldehyde-agarose gels and transferred to nylon membranes. Membranes were probed with Gαl6 (*a*) and Gαy (*b*) ^{32}p-labeled cDNA. (*a*) A 2.2-kb band was detected by the Gαl6 cDNA probe only in human kerationcytes. (*b*) A 4-kb band was detected by the Gαy cDNA probe in all four cell types. EB, ethidium bromide; HDF, human dermal fibroblasts; HK, human keratinocytes; MEC, microvascular endothelial cells; MEL, melanocytes.

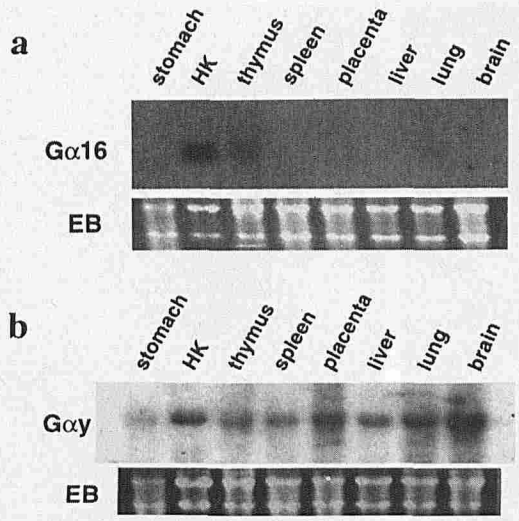

Figure 3. Northern analysis of human tissues and cultured human keratinocytes. Total RNA from normal human tissues and from cultured human keratinocytes was fractionated over formaldehyde-agarose gels and transferred to nylon membranes. Membranes were probed with Gαl6 (*a*) and Gαy (*b*) ^{32}p-labeled cDNA. (*a*) Gαl6 was clearly detected in human kerationcytes and thymus, and weakly in lung and placenta. (*b*) Gαy was detected in all tissues. EB, ethidium bromide; HK, human keratinocytes.

the other cell types (**Fig 2*a***). Gαy was expressed in all cultured cells with a message size of ≈4 kb (**Fig 2*b***). Northern analysis of normal human tissues was also performed with Gα16 demonstrating expression in thymus (**Fig 3*a***) and on longer exposure expression was also detected at low levels in lung and placenta, but no significant expression was found in brain, liver, spleen, or stomach. Gαy was expressed at varying levels in all tissues tested, with the highest expression in brain (**Fig 3*b***).

In situ* hybridization confirmed expression of Ga16 in keratinocytes *in vivo Serial tissue sections from normal baboon skin were examined by *in situ* hybridization using riboprobes prepared from the PCR-generated Gα16 and Gαy cDNA clones. Gα16 and Gαy were detected in the epidermis and hair follicle epithelium (**Figs 4*a,b*; 5*a–d***). No stratification of expression was noted within epidermal layers. In addition, Gαy, but not Gα16, was detected in arrector pili smooth muscle cells (**Fig 6*a,b***). Gαy was clearly detected in sebaceous glands and eccrine glands, but minimal if any staining over background was seen with Gα16 in these same structures (**Figs 5*a,b*; 6*c,d***). Gαy

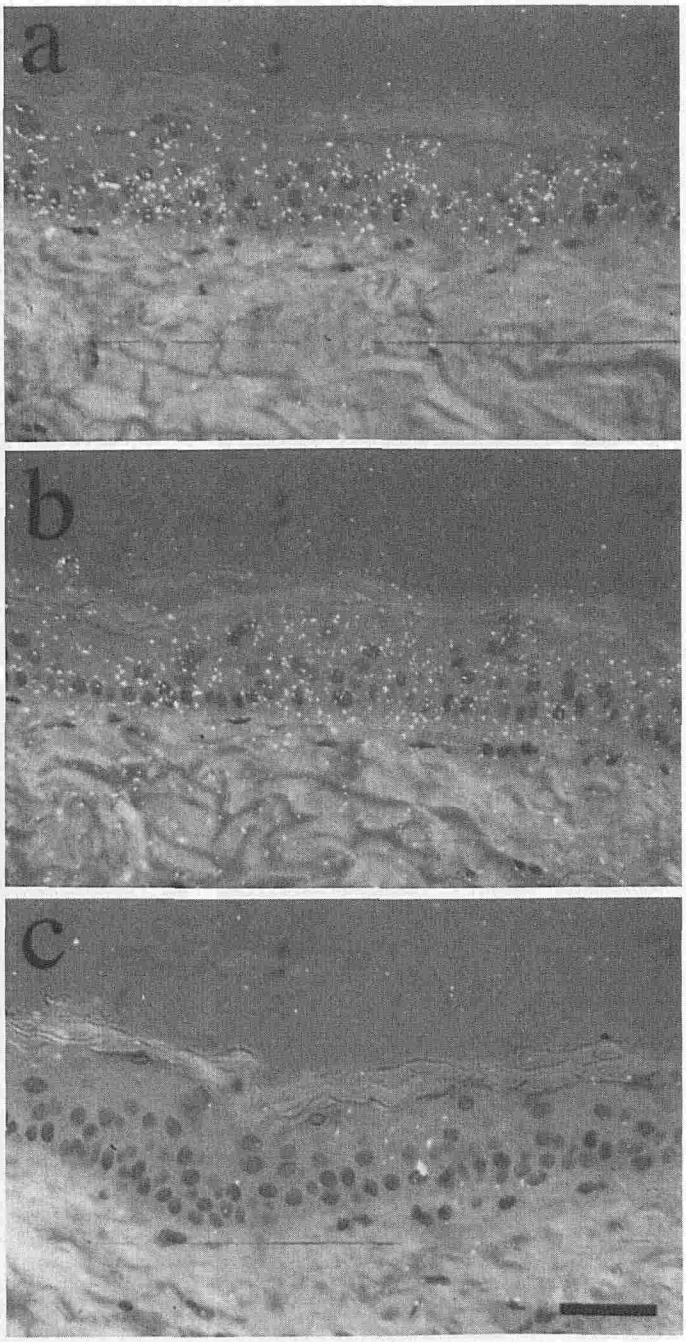

Figure 4. *In situ* hybridization of normal skin. ^{35}S-labeled riboprobes to Gαl6 and Gαy were prepared and used as probes to detect mRNA expression in normal mammalian epidermis. (*a*) Gαl6 is detected in epidermal keratinocytes. (*b*) Gαy is detected in epidermal keratinocytes. (*c*) No detection of signal is seen in the negative control, VWF. Sections were photographed with polarized light epiluminescence (Leitz) such that the silver grains appear as white spots. *Scale bar*, 50 μm.

expression was also detected in vascular structures, but Gα16 was not (**Fig 5*d,e***).

DISCUSSION

We have amplified two Gq class Gα genes, Gα16 and Gαy, from keratinocytes, cloned the PCR-generated cDNA segments and determined their expression in multiple human cells and tissues. Signaling through both of these G proteins results in phosphoinositide hydrolysis via PLC-β1, PLC-β2, and PLC-β3 with the generation of IP3 and DG second messengers (Wu *et al*, 1992). Of these, Gαy is expressed in many normal cells and tissues as we have demonstrated (**Fig 3*b***). Gαy was originally cloned from retinal epithelial cells, has an mRNA

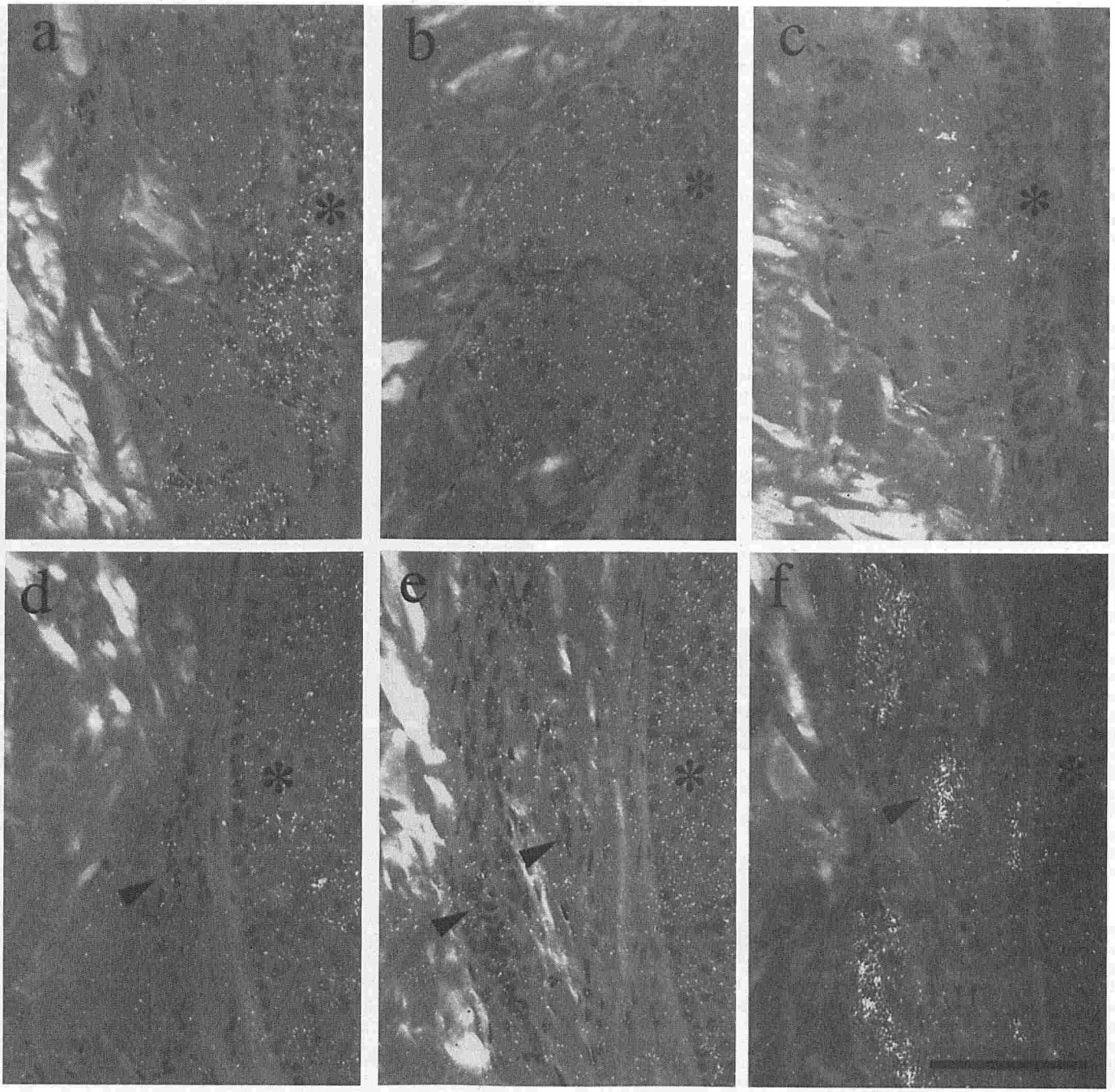

Figure 5. *In situ* hybridization of normal skin appendageal structures. ^{35}S-labeled riboprobes to Gαl6 and Gαy were prepared and used as probes to detect mRNA expression in normal mammalian dermal structures. mRNA expression is detected in the hair follicular epithelium (*) with Gαl6 (*a, d*) and Gαy (*b, e*) but not the von Willebrand factor negative control (*c, f*). Gαl6 is not significantly expressed in sebaceous glands (*a*) or vascular structures (*arrowheads*) (*d*), whereas Gαy is expressed in both sebaceous glands (*b*) and vascular structures (*e*). The VWF control is detected in endothelial cells (*f*), but not in sebaceous glands (*c*). *Scale bar*, 100 μm.

of ≈4 kb, and encodes a 359 amino acid protein with a calculated molecular weight of 42,160 (Jiang *et al*, 1991). Gαy appears to be the human homologue of the murine Gα11 (Strathmann and Simon, 1990). We have demonstrated that Gαy is expressed in most resident cells in the skin including keratinocytes, fibroblasts, melanocytes, and microvascular endothelial cells (**Fig 2*b***). Gαy expression was also detected in dermal vasculature and adnexal structures by *in situ* hybridization (**Figs 4–6**). Gαy undoubtedly plays a role in keratinocyte biology as it does in other tissues, but its ubiquitous distribution suggests that it may not play a keratinocyte-specific role.

Detecting Gα16 in keratinocytes was unexpected because of its reported restriction to human cell lines of the hematopoietic lineage, specifically of the myelomonocytic and T-cell phenotype. Therefore this finding was confirmed by cloning and sequencing a long segment from a human keratinocyte cDNA library using the Gα16 PCR-generated clone as probe. In contrast to Gαy, Gα16 has limited expression in human tissues (**Fig 3*a***). Gα16 mRNA was found only in keratinocytes cultured from neonatal foreskin and not in fibroblasts, melanocytes, or endothelial cells (**Fig 2*a***). Strong expression was detected in only epidermis and hair follicular epithelium by *in situ* hybridization, and not in other skin appendageal structures (**Figs 5, 6**). This limited expression suggests a unique keratinocyte-specific function for Gα16 in the skin, analogous to other G proteins with limited distribution such as the sensory receptor associated G protein transducin in the retina (Fong, 1992) and the olfactory neuronee specific G protein, G_{olf} (Jones and Reed, 1989). Gα16 was originally cloned from HL-60 cells (Amatruda *et al*, 1991). Gα16 expression is found primarily in undifferentiated cells as it has been shown to be

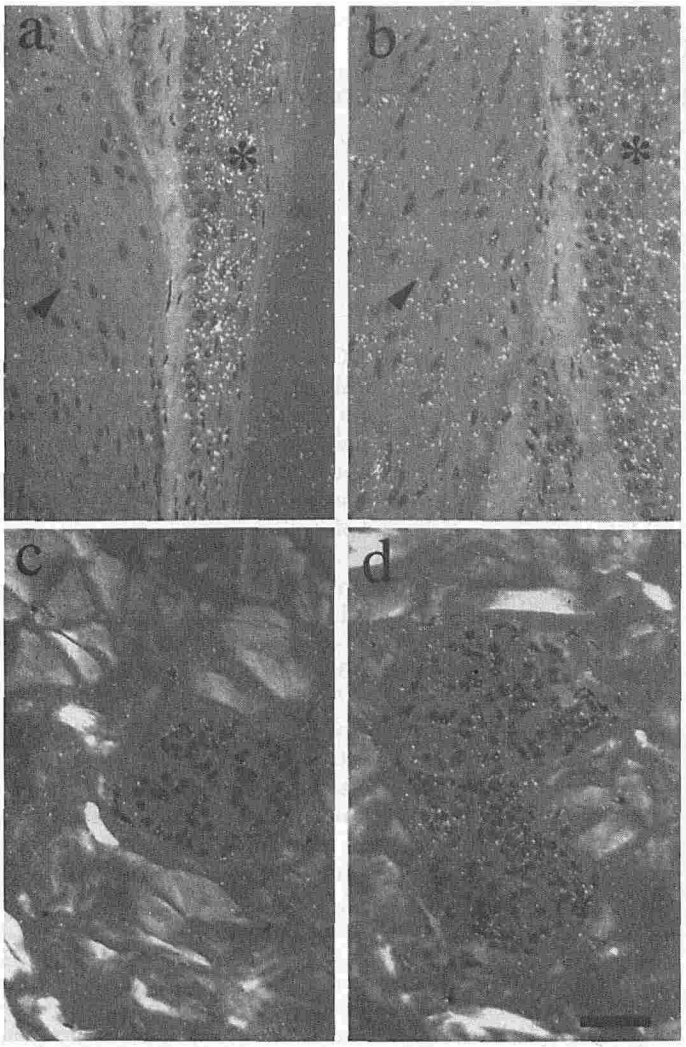

Figure 6. Gal6 is not expressed in cutaneous smooth muscle or eccrine glands by *in situ* hybridization. ^{35}S-labeled riboprobes to Gαl6 and Gαy were prepared and used as probes to detect mRNA expression in normal mammalian dermal structures. (*a*) No mRNA expression over background of Gαl6 is detected in the arrector pili smooth muscle (*arrowheads*). (*b*) Gαy mRNA is detected in the arrector pili smooth muscle. (*c*) Gαl6 is poorly if at all expressed in eccrine structures. (*d*) Gαy mRNA is detected in eccrine structures. *Scale bar*, 50 μm.

downregulated in HL-60 cells induced to differentiate to neutrophils (Amatruda *et al*, 1991). Gα16 can activate PLC-β1, 2, and 3, but is most efficient in activating PLC-β2 (Lee *et al*, 1992). The proinflammatory chemokine C5a receptor has been determined to be the physiologic receptor association for Gα16 in HL-60 cells (Amatruda *et al*, 1993; Lee *et al*, 1995), but recent evidence suggests that, *in vitro* in experimental systems, multiple seven transmembrane receptors can transduce through Gα16, but not other Gq class G proteins, and be coupled to phosphoinositide hydrolysis (Offermanns and Simon, 1995; Wu *et al*, 1995). This promiscuity of Gα16 is unusual for G proteins that normally have limited and specific receptor associations. It is not known if *in vivo* Gα16 retains this promiscuity in intact animals. Keratinocytes do not have C5a receptors, but they do have other chemokine receptors, e.g., the IL-8 receptor (Kemeny *et al*, 1995), which could be associated with this G protein and participate in inflammatory reactions in the skin such as psoriasis (Lemster *et al*, 1995).

Gα16 amino acid sequences have several distinctive features (Amatruda *et al*, 1991): Proline is present at amino acid 50 within the conserved "A" region instead of alanine or threonine. This difference has been hypothesized to play a role in the decreased GTPase activity and guanine nucleotide binding capacity in Gq classes of G proteins. There is a conserved arginine residue homologous to the cholera toxin ADP ribosylation site in G_s at amino acid 186. As with other Gq class Gα subunits, Gα16 lacks the cysteine residue at four amino acids from the carboxy terminus and is therefore not a predicted substrate for pertussis toxin ADP-ribosylation conferring its insensitivity to that toxin. Gα16 lacks a glycine at amino acid position 2 and is therefore not a predicted substrate for N-myristoylation. Gα15 is the murine homolog of Gα16 but shares only 85% amino acid identity that is unusual for G proteins that tend to be highly conserved among species. This is a unique feature of Gα16 and suggests species as well as tissue specific functions.

Gα16 is a hematopoietic and, as we have shown, keratinocyte specific G protein of the Gq class. Gα16 is likely to function in inflammatory reactions in the skin if it indeed transduces signals from a chemokine receptor as it does in HL-60 cells. The determination of the significance of Gα16 in skin will depend on elucidation of the specific physiologic receptor(s) to which it is linked in keratinocytes. As a unique potential modulator of cutaneous inflammation, Gα16 is a potential target for drug therapy.

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